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Effects of Hormone Replacement Therapy on Reactivity of Atherosclerotic Coronary Arteries in Cynomolgus Monkeys

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Objectives. We attempted to determine whether continuous and cyclic medroxyprogesterone acetate modulates the effects of estrogen on dilation of atherosclerotic coronary arteries in surgically postmenopausal female monkeys.

Background. Estrogen replacement in postmenopausal women preserves normal dilator responses of atherosclerotic coronary arteries. The effects of progestins on coronary artery reactivity have not been determined.

Methods. Repeated quantitative coronary angiography was used to study the effects after 1 month of 1) no hormone replacement (control) or oral administration of 2) continuous conjugated equine estrogens, 3) cyclic high dose medroxyprogesterone acetate (MPA) given on days 16 to 26 of the month, 4) conjugated equine estrogens plus continuous low dose MPA, or 5) conjugated equine estrogens plus cyclic high dose MPA on endothelium-mediated dilation of atherosclerotic coronary arteries in 12 cynomolgus monkeys. Change in diameter of the left circumflex coronary artery was measured in response to intracoronary infusions of acetylcholine (10^{-6} mol/liter per min) and nitroglycerin (15 μg/min).

Results. Coronary arteries constricted during no hormone treatment (−8 ± 3% [mean ± SEM]), dilated during conjugated equine estrogen treatment (+3 ± 1%, $p < 0.05$ vs. control) and constricted during cyclic MPA treatment (−3 ± 2%). Addition of cyclic or continuous MPA to the conjugated equine estrogen regimen inhibited acetylcholine responses by 50% ($p < 0.05$ vs. conjugated equine estrogens). There was no effect of treatment on vascular response to nitroglycerin ($p > 0.05$).

Conclusions. Treatment with conjugated equine estrogens, but not MPA, augmented endothelium-mediated dilation of atherosclerotic coronary arteries. Addition of cyclic or continuous MPA to the conjugated equine estrogen regimen diminished endothelium-mediated dilation.

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Estrogen replacement therapy augments endothelium-mediated dilation of atherosclerotic coronary arteries of postmenopausal women (1) and monkeys (2,3). The effects of estrogen on coronary artery reactivity may be one mechanism by which estrogen reduces coronary heart disease risk in postmenopausal women. Indeed, results of a recent study (4) indicated that sublingual administration of estradiol in women with coronary heart disease reduced myocardial ischemia during exercise treadmill tests.

A progestin usually is added to estrogen replacement regimens to reduce the increased risk of endometrial cancer associated with unopposed estrogen. One of the common progestins, medroxyprogesterone acetate (MPA), is given continuously at a low dose (2.5 mg/day) or cyclically (days 16 to 26 of the menstrual cycle) at a higher dose (10 mg/day). It has been suggested (5,6) that progestins increase the risk of coronary heart disease in women by decreasing plasma concentrations of high density lipoprotein (HDL) cholesterol. However, other mechanism(s) by which progestins increase the risk of coronary heart disease remain unclear. Parenterally administered progesterone does not have an adverse effect on progression of coronary artery atherosclerosis in monkeys when combined with parenterally administered estradiol (7). However, no studies have examined the effects of traditional oral progestin replacement regimens on reactivity of atherosclerotic coronary arteries. Therefore, ovariectomized monkeys were used to examine the separate and combined effects of oral conjugated equine estrogens and continuous low dose or cyclic high dose MPA on dilator responses of atherosclerotic coronary arteries.

Methods

Twelve adult female cynomolgus monkeys (*Macaca fascicularis*), 8 to 12 years old, were fed a semipurified diet ad libitum for 24 to 36 months that contained 0.4 mg of cholesterol/calorie and 40% of calories as fat. The diet results in plasma cholesterol concentrations of 8.5 to 10 nmol/liter and induces moderate amounts of coronary artery atherosclerosis in 2 to 3 years.

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Experimental design. The experiment utilized a repeated-measures design in which all monkeys received all treatments for 1 month. One month of treatment was chosen because it was thought that each monkey should receive at least one "cycle" of treatment, and estrogen has been shown to affect coronary artery reactivity within minutes (3). There was no washout time between treatments. However, monkeys received treatments in a random order to minimize any time or carryover effect of treatment on vascular reactivity.

The monkeys were not euthanized. Therefore, atherosclerosis extent was not measured. We reported previously (2) that female monkeys fed atherogenic diets for this period of time develop moderate amounts of coronary artery atherosclerosis. With this amount of atherosclerosis, estrogen exerts an effect on coronary artery vasomotion independent of plaque size (2). Therefore, it is unlikely that individual differences in atherosclerosis affected experimental results. Because treatments were randomized, it is unlikely that progression or regression of coronary artery atherosclerosis during the experiment affected experimental results.

The treatments were as follows: 1) no hormone replacement; 2) conjugated equine estrogens (Premarin, Wyeth-Ayerst) given continuously in the diet at a dose (0.11 mg/day) equivalent to 0.625 mg of conjugated equine estrogens/day in women; 3) cyclic "high dose" MPA (Cycrin, Wyeth-Ayerst) given in the diet on days 16 to 26 of the treatment period at a dose (0.42 mg/day) equivalent to 10 mg of MPA/day in women; 4) combined conjugated equine estrogens and cyclic high dose MPA; and 5) combined conjugated equine estrogens and "low dose" continuous MPA (0.1 mg/day) equivalent to 2.5 mg of MPA/day in women.

Laboratory measurements. Blood samples were collected from anesthetized monkeys at baseline, before any treatment was initiated and on day 26 of each treatment period. Monkeys were anesthetized with ketamine hydrochloride (10 to 15 mg/kg body weight intramuscularly) and butorphanol (0.025 mg/kg intramuscularly). After blood was obtained by venipuncture, plasma was separated by centrifugation for lipid concentration determinations. Concentrations of total plasma and HDL cholesterol were determined by previously described methods (8,9).

Blood samples for plasma concentrations of estradiol (a metabolite of conjugated equine estrogens) and MPA were analyzed according to previously described methods (10,11).

Coronary angiography. On day 26 of each treatment period, coronary angiography was performed to measure the effect of the hormone replacement regimens on endothelium-mediated and smooth muscle-mediated dilation of the left circumflex coronary artery. Coronary angiography was performed immediately after taking blood samples for plasma lipid and sex hormone determinations. Supplemental doses of ketamine and butorphanol were given to maintain light surgical anesthesia. Monkeys were allowed to breathe spontaneously and were warmed by a circulating water blanket. The use of quantitative coronary angiography to measure coronary artery reactivity has been described elsewhere and validated in female cynomolgus monkeys (2,3).

Coronary artery diameter was measured during intracoronary infusion of 1) 5% dextrose in water (control diameter); 2) 10^{-8}, 10^{-7} and 10^{-6} mol/liter of acetylcholine (final concentration in the coronary artery); and 3) nitroglycerin (15 μg/min). Coronary artery reactivity was measured as percent change in diameter compared with the diameter measured during the control infusion.

The catheters were removed at the end of the study, and each monkey was allowed to recover from anesthesia. Postoperative care included monitoring the monkeys until they were fully awake and giving supportive care if needed. None of the monkeys required postoperative antibiotics or pain medication. All procedures were carried out in accordance with state and federal regulations and were approved by the Institutional Animal Care and Use Committee.

To determine the accuracy and precision of the quantitative coronary angiography methods used, a number of additional analyses were performed. Images of a plexiglas phantom with five precision-drilled holes ranging in diameter from 0.73 to 4.79 mm were obtained under radiograph conditions (K.V.P. and M.A.) similar to those in monkey angiography. The correction coefficient was 0.99, with a standard error of 0.05 mm. For analysis of monkey angiograms, each film was analyzed in an identical manner on two separate occasions by an operator who was unaware of the initial results or the treatment groups of the monkeys. The average of these two measurements (360 measurements made on two occasions) was used for the analysis. The correlation between repeated measures was 0.98, and the mean difference between measures was -0.002 ± 0.028 mm. The mean absolute difference was 0.04 mm, and the coefficient of variation was 2.4%.

Statistics. Data shown are mean value ± SEM. If data were not distributed normally, they were first subjected to linear transformation. The effect of treatment on experimental variables (plasma lipids and coronary artery reactivity) was analyzed using repeated-measures analysis of variance. Post hoc analyses were by Duncan's multiple comparison procedure; $p < 0.05$ was considered significant.

Results

Laboratory measurements. Total plasma and HDL cholesterol concentrations were similar at baseline (before any treatment) and during the no-hormone replacement phase (control) of the experiment regardless of when the no-hormone replacement occurred in the order of treatment ($p > 0.05$) (Table 1). There were no treatment effects on total plasma or HDL cholesterol (for both, $p > 0.05$) (Table 1).

Plasma estradiol and MPA concentrations were similar at baseline and during control infusion ($p > 0.05$) (Table 1). As expected, plasma estradiol concentrations were higher than control during treatment with conjugated equine estrogens or conjugated equine estrogen plus MPA treatments ($p < 0.05$ vs. control) (Table 1). Plasma concentrations of MPA were similar at baseline and during control ($p > 0.05$) (Table 1). Additionally, plasma concentrations of MPA were higher than control

Table 1. Effects of Treatment on Plasma Lipid, Sex Hormone Concentrations and Lower Doses of Acetylcholine and Nitroglycerin (mean ± SEM)

	Baseline	Control	CEE	MPA	CEE+Cyclic MPA	CEE+Cont MPA
TPC (mmol/liter)	9.3 ± 0.8	9.1 ± 1.1	9.6 ± 1.1	8.8 ± 1.3	10.1 ± 0.5	10 ± 0.7
HDL-C (mmol/liter)	1.2 ± 0.1	1.1 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	1.2 ± 0.3
Estradiol (pmol/liter)	15.9 ± 3.2	22.2 ± 6.5	343 ± 47.7*	60.4 ± 19.1*	321 ± 38.2*	359 ± 31*
MPA (pmol/liter)	BDL	BDL	BDL	238 ± 27*	195 ± 31*	65 ± 20*†
% change in diameter after						
Acetylcholine (10^{-6} mol/liter)	0 ± 2	1 ± 1	−2 ± 2	1 ± 1	0 ± 1	−3 ± 2
Acetylcholine (10^{-7} mol/liter)	−1 ± 1	−1 ± 2	−5 ± 3	−3 ± 2	0 ± 2	−2 ± 3
Nitroglycerin (15 μg/kg per min)	10 ± 3	9 ± 2	11 ± 3	13 ± 2	10 ± 3	12 ± 1

*p < 0.05 versus control, †p < 0.05 versus conjugated equine estrogens (CEE) plus cyclic medroxyprogesterone acetate (MPA) group. BDL = below detectable limits; Cont = continuous; HDL-C = high density lipoprotein cholesterol; TPC = total plasma cholesterol.

during MPA or conjugated equine estrogen plus MPA treatments ($p < 0.05$ vs. control) (Table 1). Plasma concentrations of MPA were higher during high dose than low dose MPA treatments ($p < 0.05$) (Table 1).

Coronary angiography. The basal diameters of coronary arteries (before any of the treatment regimens) was 1.3 ± 0.2 mm. Basal diameters did not change significantly during the different interventions ($p > 0.05$). Results of 10^{-7} and 10^{-8} mol/liter of acetylcholine on coronary artery reactivity were not significant ($p > 0.05$) (Table 1). Coronary arteries constricted to 10^{-6} mol/liter of acetylcholine when monkeys received no hormonal replacement but dilated during conjugated equine estrogen treatment ($p < 0.05$ vs. control) (Fig. 1). Treatment with high dose cyclic MPA did not significantly improve coronary artery response to acetylcholine ($p > 0.05$ vs. control) (Fig. 1). However, addition of cyclic high dose or continuous low dose MPA to conjugated equine estrogen treatment diminished the beneficial effect of conjugated equine estrogens on acetylcholine-induced dilation of atherosclerotic coronary arteries ($p < 0.05$ vs. conjugated equine estrogens) (Fig. 2). There were no treatment effects on dilator responses to nitroglycerin (for all, $p > 0.05$ vs. control) (Table 1).

Discussion

The four major findings of this study were that among surgically postmenopausal female monkeys with diet-induced coronary artery atherosclerosis, 1) oral administration of conjugated equine estrogens improved endothelium-mediated dilation of coronary arteries; 2) oral administration of a progestin did not improve impaired endothelium-mediated dilation; however, 3) addition of a progestin (given cyclically or continuously) to the estrogen regimen diminished the beneficial effects of unopposed estrogen on endothelium-mediated dilation; and 4) oral administration of an estrogen or progestin, or both, had no effect on endothelium-independent dilation of atherosclerotic coronary arteries.

Repeat coronary artery angiography allows repeated measures of coronary artery reactivity within the same monkey and increases the power of observations. Because there was no

Figure 1. Effects of conjugated equine estrogens (CEE) and medroxyprogesterone acetate (MPA) on coronary artery response to acetylcholine. Results presented are mean value ± SEM change in coronary artery diameter of the left circumflex coronary artery compared with that after infusion of 5% dextrose in water. *p < 0.05 versus no hormone replacement (control).

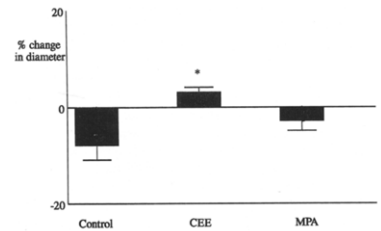
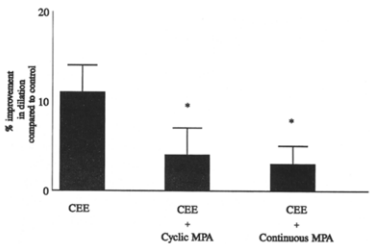


Figure 2. Effects of added cyclic medroxyprogesterone acetate (MPA) and continuous MPA on coronary artery reactivity to acetylcholine in monkeys receiving conjugated equine estrogens (CEE). Results are expressed as percent change in diameter of the left circumflex coronary artery during treatment minus percent change in diameter during no hormone replacement. *p < 0.05 versus conjugated equine estrogens.



washout period between treatments, the order of treatments was randomized to reduce the possibilities that 1) the effects of treatment on coronary artery reactivity were due to residual effects of the previous treatment; 2) progressive damage to the coronary artery endothelium by the catheter affected coronary artery reactivity; and 3) progression of coronary artery atherosclerosis during the experiment was not a variable affecting coronary artery reactivity. The belief that residual effects of treatment on coronary artery reactivity were minimal is supported by the finding that there were no differences in plasma lipoprotein, estradiol or MPA concentrations during baseline (before any treatment) and control (done at varying times during the experiment). Furthermore, this finding indicates that 1 month was a sufficient time to wash out the previous treatment.

Unlike the present study, earlier studies (1–3) have examined the effects of parenteral administration of estrogen (or grouped oral and parenteral administration of estrogen) on endothelium-mediated dilation of coronary arteries. Results of the current study extend those of previous studies by examining the separate and combined effects of oral administration of conjugated equine estrogens and a progestin on coronary artery reactivity. Importantly, the present study examines the effects of two common progestin regimens (cyclic and continuous MPA) on coronary artery reactivity in monkeys receiving conjugated equine estrogens.

Effects of estrogen. Long-term (33 month) subcutaneous administration of 17-beta estradiol to postmenopausal monkeys improves endothelium-mediated dilation of atherosclerotic coronary arteries (2). Furthermore, endothelium-mediated dilation of atherosclerotic coronary arteries is improved 20 min after intravenous infusion of ethinyl estradiol (3). This effect of estrogen replacement on endothelium-mediated dilation of atherosclerotic coronary arteries has been confirmed in postmenopausal women (1). In our previous studies (12), estrogen was given at physiologic doses that resulted in plasma estradiol concentrations similar to those of peak follicular phase estrogen (~920 pmol/liter). Pharmacologic doses of 17-beta estradiol have been shown to improve endothelium-independent dilation of rabbit coronary arteries studied in vitro, possibly by activating ion channels (12). The results of the present experiment support those of previous studies by showing that physiologic doses of oral conjugated equine estrogens improve endothelium-mediated dilation of atherosclerotic coronary arteries studied in vivo. However, it remains unclear why this is so, when by contrast, high doses of estrogen affect endothelium-independent dilation of nonatherosclerotic arteries when studied in vitro. Perhaps the dose of estrogen or the disease state of the artery affects estrogen-mediated vascular responses of arteries.

The magnitude of improvement in endothelium-mediated dilation reported in the present experiment (11%) was within the sensitivity range of the quantitative angiography methods but was less than in previous experiments that showed a 30% to 35% (2) and 21% (3) improvement after parenteral administration of estradiol. The physiologic importance of these smaller improvements in response to oral conjugated equine estrogens is unclear. Acetylcholine is a test substance only. Any improvement or impairment of dilation due to acetylcholine can be interpreted only as an index of coronary artery function. In this sense, any improvement or impairment in coronary function could be physiologically important in the pathogenesis of coronary heart disease. The dose of conjugated equine estrogens in the present experiment resulted in lower plasma estradiol concentrations than previous experiments (320 vs. 920 pmol/liter, respectively). Parenteral administration of estradiol is likely to provide continuous and consistent blood estrogen concentrations, whereas oral administration of conjugated equine estrogens may result in inconsistent and widely varying blood levels of estradiol dependent on the intake of food and bioavailability. In addition, conjugated equine estrogens contain many different estrogens. It remains undetermined whether some estrogens are more potent than others in their ability to act on coronary arteries. Therefore, dose of estrogens, route of administration and differing potency of estrogens may explain differences in coronary artery responses.

Effects of progestins. The public health concern that progestins may adversely affect the cardiovascular system is based on the finding that progestins, when coadministered with estrogens, decrease plasma concentrations of HDL cholesterol (5). However, there are few direct experimental data regarding the effects of progesterone or progestins on coronary artery structure or function. It has been shown (7) that addition of progesterone to an estrogen replacement regimen does not worsen the extent of coronary artery atherosclerosis among postmenopausal monkeys. Furthermore, pharmacologic doses of progesterone improve endothelium-mediated dilation of rabbit coronary arteries studied in vitro (13). In the present experiment, cyclic high dose and continuous low dose MPA treatment resulted in plasma MPA concentrations of ~200 and 30 pmol/liter, respectively. These high dose plasma concentrations are somewhat lower than those achieved in women (300 and 100 pmol/liter, respectively). It is unknown whether higher plasma MPA concentrations in monkeys would have had more profound effects (i.e., inhibition of estrogens) on vascular reactivity. The lower MPA concentrations in monkeys would not be recommended in women with a uterus because of incomplete endometrial protection against cancer.

It is unclear whether it is reasonable to compare the cardiovascular effects of progesterone with those of other progestins. Haarbo et al. (14) administered two different 19-nortestosterone–derived progestins to surgically postmenopausal rabbits fed an atherogenic diet. In that study, the progestins did not affect HDL cholesterol, and when given in combination with estradiol did not diminish the beneficial effect of estradiol on development of aortic atherosclerosis. Furthermore, micronized progesterone does not have an HDL cholesterol-lowering effect in women (15). In the present experiment, MPA was chosen because it is the progestin most commonly prescribed in postmenopausal hormone replacement regimens in the United States. Administration of MPA did not affect total plasma or HDL cholesterol concentrations

when given alone or in combination with conjugated equine estrogens. However, MPA did diminish the beneficial effect of conjugated equine estrogens on endothelium-mediated dilation of atherosclerotic coronary arteries. This effect was observed whether MPA was given cyclically at a relatively high dose (10 mg/day) or continuously at a lower dose (2.5 mg/day).

Our results can be reconciled with those that have indicated a harmful effect of progestins on plasma lipids. In women (6) and monkeys (7), sex hormone replacement effects on plasma lipids explain only 30% of the beneficial effects of estrogen on risk of coronary artery disease. Similar results are reported (16) in premenopausal monkeys given oral contraceptives with the same amounts of ethinyl estradiol but different progestins. Despite an HDL cholesterol-lowering effect of progestins, coronary artery atherosclerosis was less in treated than untreated animals. Therefore, the changes in lipids caused by sex hormones (given premenopausally or postmenopausally) appear to play a minor role in the pathogenesis of coronary heart disease compared with direct effects of sex hormones on the artery wall.

Conclusions. We believe that the results of this experiment are the first to show that conjugated equine estrogens, like parenterally administered estradiol, induce positive effects on vasomotion of atherosclerotic coronary arteries. Addition of a progestin diminished these positive effects, raising the possibility of a potentially clinically harmful effect of progestin replacement in postmenopausal women.

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